STUDIES ON PNEUMOCOCCUS GROWTH INHIBITION.

I. The Protective Action of Gelatin for Pneumococci in Suspension.

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During the course of certain experiments on the inhibition of the growth of pneumococci in a serum-leucocyte mixture, which are presented in detail in the second paper of these studies, there occurred certain irregularities in growth that seemed to be traceable to variations in the pneumococcus suspension used. At times growth failed to take place in the control tubes where it should have occurred had the organisms been present in the calculated numbers or in a suitable condition at the time of seeding. The method of making up the original suspension appeared to preclude much variation in numbers, and great care was taken to use organisms during their active growth phase. The probability was, therefore, that the solution used for suspension had an injurious effect on the organisms.

In these experiments Locke's solution was employed instead of the customary normal salt solution, because of Shearer's (1) findings that pneumococci (in common with several other organisms studied) when suspended in 0.85 per cent NaCl solution, showed evidence of early injury which resulted in the death of the organisms after a few hours. If, on the other hand, a balanced Ca-Na solution[1] was used as the suspension fluid, the organisms could be maintained in an apparently uninjured state for relatively long periods of time. Shearer, however, worked with large numbers of bacteria, while in the present experiments minute quantities were employed—0.0000001 cc. of a suspension containing approximately 1,000 million pneumo-

[1] Shearer used Ringer's solution.

cocci per cc. The use of such small numbers of organisms constitutes a much more severe test of the suitability of the suspension fluid. The death of a few hundred or thousand organisms in a heavy suspension would make no appreciable difference in its viability, while the death of a small number in a suspension containing only 50 or 100 organisms per unit of solution might result in a sterile suspension.

The Locke's solution employed was adjusted to pH 7.8 to 8.0, since it was assumed that the hydrogen ion concentration found to be most favorable for the growth of pneumococci (Dernby and Avery (2)) was also the most suitable for suspension in a non-nutrient fluid. However, in view of the irregularities in growth cited above, it seemed important to investigate the effect of varying the reaction of the Locke's solution. Furthermore, the possibility existed that the presence of a small quantity of protein in Locke's solution, or in other crystalloid solutions, might add something to their protective value. Accordingly, the following experiments were undertaken, with the purpose of determining the type of non-nutrient fluid most suitable for the suspension of pneumococci with a minimum amount of injury.

Standardization of the Pneumococcus Suspension.

1. Number of Organisms.—In order to obtain comparative results with the different solutions used, it was necessary to add the organisms in constant quantities. For the purpose of standardizing the suspension of pneumococci, Gates' (3) method of measuring the opacity of bacterial suspensions was employed. By means of bacterial counts it was determined that a suspension containing approximately 1,000 million pairs of pneumococci gave a corrected reading (Gates) of 2.1. In the following experiments the standard suspension of pneumococci used was always one with an opacity represented by the corrected reading 2.1.

2. Culture Media.—The culture medium used for growing the organisms for the suspension was meat infusion broth having a pH of 8.0. In order to obtain a vigorously growing culture it was found necessary to enrich the medium by adding 0.05 to 0.1 per cent dextrose[2] and 2 to 3 per cent rabbit serum.

3. Growth Phase.—Suspensions were always made from organisms in the active growth phase. To prepare the culture for the test, three tubes of serum broth (5 cc.), to which 0.05 per cent dextrose had been added, were inoculated with a loopful, 0.05 and 0.01 cc., respectively, of the stock culture (blood broth) and allowed to incubate overnight, usually for 14 to 15 hours. The tube showing the most active growth was used for subculturing. 1 to 5 cc. of culture, the amount

[2] The broth medium made with meat obtained in Peking is poor in sugar.

depending on the time at which it was desired to make up the suspension, were transplanted into 25 cc. of the serum-dextrose (0.1 per cent) broth contained in a 50 cc. centrifuge tube. If the broth has been kept in the ice box it is advantageous to take off the chill before inoculation with culture. Growth was allowed to proceed to the point at which transparency of the contents had almost disappeared. The tube was then centrifuged at high speed for 20 minutes and the supernatant fluid removed. The sediment was taken up in 4 to 5 cc. of the solution to be used for suspension and after thorough mixing, opacity readings were made and the standard suspension prepared.

4. Dilutions.—To make up the dilutions of the pneumococcus suspension, 1.8 cc. of the diluting fluid was placed in each of six test-tubes (15 mm. × 15 cm.). 0.2 cc. of the standard suspension, well mixed previously, was then added to the first tube and after thorough mixing, 0.2 cc. from each tube was successively added to the next, thereby making a series of dilutions ranging from 0.01 to 0.0000001 cc. of the standard, per 0.1 cc. of the dilution, or, in numbers, from approximately 10 million to 100 organisms. In order to make the dilutions as accurate as possible the following technique was employed. The same pipette was always used through the procedure. Fluid from the standard suspension was drawn up to the 0.7 mark on the pipette or slightly above this level, care being taken not to raise up the column of fluid higher than the 0.6 mark. 0.2 cc. was then delivered into the first tube from the 0.7 to the 0.9 marks and the remainder returned to the standard suspension tube. Thorough mixing of the tube's contents was obtained by tapping the bottom of the tube against the palm of the hand with a rotary motion. The first three tubes of each dilution were shaken for 3 minutes, and the last three for 4 minutes each, the pipette meanwhile being held in the mouth near the upper end. Before using the pipette for the next dilution it was rapidly passed through the flame once or twice, with a rotary motion. In order to prevent carrying over to the second dilution some of the standard suspension which might still be adhering to the inner wall of the pipette, the first dilution (before transfer) was drawn up several times to a point —the 0.5 mark or thereabouts—well above the level to which fluid from the previous concentration had reached. This process was repeated before each successive transfer—*i.e.*, each time the pipette was washed to a higher level than the time before—but the 0.2 cc. was always delivered from the 0.7 to the 0.9 marks. The dilutions were not made up until just before they were needed for the test. This precaution was probably not necessary, since it has been found that suspension in a suitable fluid, for several hours at least, probably does not injure the pneumococcus.

5. Organism Used.—The strain of organisms used in these experiments was a Type I pneumococcus, isolated 4 years ago from a case of lobar pneumonia, kept since in blood broth. Just before the beginning of these tests it was passed rapidly through four guinea pigs. It grew readily in artificial media; 0.0000001 cc. of the standard suspension, diluted in a suitable suspension fluid, and planted in dextrose blood broth, gave an abundant macroscopic growth in 12 hours. The organism was highly virulent for rabbits and mice.

Preparation of Solutions.

1. Locke's Solution.—The Locke's solution was made up as follows:

NaCl	0.9	gm.
$CaCl_2$	0.024	"
KCl	0.042	"
$NaHCO_3$	0.01 to 0.05	" *
H_2O	100.00	cc.

* Depending on the pH desired.

2. Salt Solution.—Where the per cent of NaCl is not mentioned, 0.9 per cent is indicated.

3. Gelatin Solutions.—These solutions were made by adding 0.1 per cent gelatin ("Difco bacto") before sterilization. The gelatin is most easily dissolved in distilled water heated gently and stirred during the process.

4. Water.—The water employed for making the solutions had been distilled twice in a glass still and boiled immediately before using, in order to render it CO_2-free.

5. Adjustment of Reaction.—On account of the marked changes in reaction that an adjusted solution always undergoes during sterilization, the solutions for the most part were not adjusted before autoclaving. At the beginning of the work the desired reaction was obtained by adding varying amounts of $NaHCO_3$ (from 0.2 to 1.0 cc. of a 0.5 per cent solution) before sterilization, to a series of 50 cc. quantities of the freshly prepared solution contained in small flasks. The reactions of these several flasks after autoclaving were usually found to lie between pH 7.0 and 8.0 with probably two or three of them at the H ion concentration desired. While this method obviated the necessity of adjustment after sterilization, it was uncertain and involved making up a great deal more of the solution than could be used.

Later it was found much simpler to sterilize the solution without adding $NaHCO_3$ and let the flasks stand in the ice box for several days until the exchange of CO_2 had come to an equilibrium, before adjusting. One of the flasks was then adjusted without using the gas flame, which introduces an increased concentration of CO_2 and thereby causes a change in the solution toward acidity.[3] The amount of N/10 $NaHCO_3$ found necessary to bring the contents of this flask to the desired pH was next added to each of the other flasks. These latter flasks were of course flamed on opening, in order to preserve sterility. While there is an immediate change in reaction following flaming, solutions containing bicarbonate have been found to return, after several hours, to their former H ion level. In the latter part of the work the solutions, with the exception of Locke's, were buffered after adjustment with M/15 balanced phosphate mixture (sterilized by autoclaving). The buffer was added in the ratio of 1 part of buffer to 50 or 100 parts of solution. This increased considerably the stability of the reaction.

[3] Factors influencing the degree of change in reaction due to flaming will be given in detail in a subsequent communication.

Culture Media Employed.

In testing the viability of the organisms in suspension, dextrose blood broth was used chiefly. To meat infusion broth, adjusted, with a pH of 8.0 and buffered with 0.1 per cent K_2HPO_4, 2 per cent defibrinated blood and 1 per cent dextrose were added. The broth was then distributed into test-tubes in 5 cc. quantities and incubated for 24 hours before use. A single lot of dextrose blood broth was employed in each experiment. For plating, 1 per cent dextrose rabbit blood agar, of pH 7.8 to 8.0, was used.

The technique of plating was carried out as follows: First, 1 cc. of defibrinated rabbit blood was delivered into a deep plate. Next, 0.1 cc. of the suspension to be cultured was placed in the plate near the blood, with which it was immediately mixed. Finally, 10 to 12 cc. of agar, at a temperature of 40–41°C., which had been cooled at 43° for at least 15 minutes previously, were poured into the plate and the contents well mixed by rotation. By this means a greater number of colonies was obtained than when the culture material was added to the agar in the tube, as is usually done.

The inoculated tubes and plates were always observed at frequent intervals for at least 72 hours before being considered negative.

Suspension in Locke's and Gelatin-Locke's Solutions.

Experiment 1.—(Table I.) The standard suspension was made up in gelatin-Locke's solution. From this four separate series of dilutions were made, two in Locke's and two in gelatin-Locke's solution. Immediately after the completion of the final dilution 0.1 cc. of this suspension was plated in rabbit blood agar. Two of the suspensions, one Locke's and one gelatin-Locke's, were left at room temperature; the other two were placed in the incubator. Cultures from these tubes (0.1 cc. of the suspension) after thorough agitation, were made into dextrose blood broth, pH 8.0, at short intervals of time for a period of 30 hours, as indicated in the table.

It is seen in Table I that the pneumococci suspended in gelatin-Locke's solution remained viable for a very much longer time than did those in the plain Locke's solution. How long the pneumococci would have survived in gelatin-Locke's solution at room temperature is not known, since the test was terminated at the end of 30 hours. The fact that the gelatin-Locke's suspension kept at incubator temperature yielded sterile cultures after 9 hours indicates that there could have been little if any growth in this solution.

Experiment 2.—(Table II.) The standard suspension of pneumococci was made up in gelatin-Locke's solution, pH 7.6. From this suspension dilutions

were made into gelatin-Locke's solutions ranging in pH from 7.0 to 8.0. Each dilution was shaken for only ½ to 1 minute instead of the customary 3 to 4 minutes. Cultures of the test suspensions were made as in Experiment 1, using the same test media.

TABLE I.

Survival of Pneumococci in Locke's and Gelatin-Locke's Solutions at Room and Incubator Temperatures.

Solution used.	No. of organisms.		Culture in dextrose blood broth.							
	Amount of standard suspension.	Colonies in plate.	At hrs.							
			0	3	6	9	12	19	24	30
Room temperature.	*cc.*									
Locke's pH 7.7.	0.000001		+	+	+	+	0	0	0	
" pH 7.7.	0.0000001	46	+	+	+	+	0	0	0	
Gel.-Locke's pH 7.8.	0.000001		+	+	+	+	+	+	+	+
" pH 7.8.	0.0000001	40	+	+	+	+	+	+	+	+
Incubator temperature.										
Locke's pH 7.7.	0.000001		+	0	0	0	0	0	0	
" pH 7.7.	0.0000001	38	+	0	0	0	0	0	0	
Gel.-Locke's pH 7.8.	0.000001		+	+	+	+	0	0	0	0
" pH 7.8.	0.0000001	45	+	+	+	0	0	0	0	0

TABLE II.

Survival of Pneumococci in Gelatin-Locke's Solutions of Varying Hydrogen Ion Concentration.

pH of solution.	Amount of standard suspension.	Culture in dextrose blood broth.					
		At hrs.					
		4	9	15	18	21	24
Room temperature 24–25°C.	*cc.*						
8.0	0.0000001	+	+	+	+	+	+
7.8	"	+	0	0	0	0	0
7.6	"	+	+	+	+	+	+
7.5	"	+	0	0	0	0	0
7.3	"	+	+	+	+	+	+
7.2	"	+	+	+	+	+	+
7.0	"	+	+	+	+	+	0
Incubator temperature 37°C.							
7.6	0.0000001	+	+	0	0	0	0

The results of this experiment show that the reaction of gelatin-Locke's solution may be varied over a range of pH 7.0 to 8.0 without affecting appreciably its protective properties for pneumococci. The early death in the suspensions of pH 7.8 and 7.5 could be interpreted in only one way; namely, that these two dilutions contained much fewer organisms than the others, as a result of inadequate mixture during the process of making the dilutions. Further experiments showed this assumption to be correct. It was found that when the time of agitation was shortened much below that of the standard adopted, marked irregularities in the final dilution resulted. With dilutions properly prepared, a seeding into plates of 0.0000001 cc. of the standard suspension (0.1 cc. of the sixth dilution) made in gelatin-Locke's solution, showed on the average 40 to 60 colonies. Sometimes the number was a little below or somewhat above this range.

Suspension in Salt Solution and Water with and without Gelatin.

In order to determine whether the addition of gelatin to fluids other than Locke's would likewise confer on them the same increased protective action for pneumococci shown by gelatin-Locke's solution, the following experiments were undertaken.

Experiment 3.—(Table III.) The solutions with the exception of gelatin-Locke's, were buffered with 1:100 phosphate mixture (pH 7.8). The standard suspension and the main series of dilutions were made up in gelatin-water. In order to save time the dilutions in plain water were begun from the third dilution of the gelatin-water series, *i.e.* 0.2 cc. of the third gelatin-water dilution was placed in 1.8 cc. of plain water, and the remaining three dilutions continued in this fluid. Similarly the dilutions in plain salt solution, 0.9 per cent, were begun from the third gelatin-water dilution. The other gelatin solution suspensions were begun from the fourth dilutions of the gelatin-water series. The sixth dilution in each case was made up to double the amount of the preceding dilutions in order to provide sufficient fluid for testing H ion concentration. In each case, immediately after the completion of the final dilution, 0.1 cc. of this suspension was plated in dextrose blood agar. The suspensions were then kept at a temperature of 23°C. except for the first 5 or 6 hours during which time the initial temperature of 26° dropped gradually to 23°C. At successive intervals of time, 0.1 cc. of the contents of the suspension tubes, after 3 to 4 minutes' agitation, was transplanted into dextrose blood broth. The reaction of the several fluids was determined immediately after distribution into the dilution tubes and at the end of 24 hours the H ion concentration of each suspension tube's contents was tested.

TABLE III.

Comparison of the Survival of Pneumococci in Salt Solutions and Water with and without Gelatin.

Solution used.	pH of solution at beginning.	Temperature at which suspension was maintained.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs. 0	1	3	6	9	20	24	48	72	96	120	144	168	192	pH of solution at 24 hrs.
		°C.																
NaCl 0.9 per cent.	7.8	23	0	0	0	0	0	0	0	0								8.0
Gel.-NaCl 0.9 per cent.	7.8	23	41	−	+	+	+	+	+	+	+	0	0	0	0			7.8
Water.	7.8	23	43	+	0	0	0	0	0	0								8.1
Gel.-water.	7.8	23	48	−	+	+	+	+	+	+	+	+	+	+	+	+	0	7.8
Gel.-Locke's.	7.7	23	45	−	+	+	+	+	+	+	+	+	+	+	+	0	0	7.9
NaCl 0.9 per cent.	7.8	37	0	0	0	0	0	0	0	0								8.3
Gel.-NaCl 0.9 per cent.	7.8	37	34	−	+	+	0	0	0	0								8.2
Water.	7.8	37	50	+	0	0	0	0	0	0								8.4
Gel.-water.	7.8	37	49	−	+	+	+	+	0	0								8.0

The contrast between the gelatin and non-gelatin solutions is strikingly brought out in Table III. No organisms could be recovered from the plain salt solutions at the completion of the dilutions, while suspensions in gelatin-salt 0.9 per cent solution remained alive for 48 hours. Similarly with water, pneumococci could not be recovered from the water suspension after 1 hour's standing, while those organisms suspended in gelatin-water remained viable for 7 days. It will also be noted that in gelatin-water and gelatin-Locke's solutions pneumococci remained alive three times as long as they did in gelatin-salt solution.

The fact that the reaction of the water suspension at 23°C. was found to be at pH 8.1 after 24 hours, suggested the possibility that the difference shown by this experiment between water and gelatin-water might, at least to a certain degree, be referable to the greater alkalinity of the water. However, a repetition of this portion of the experiment, in which the reaction of the solutions remained between the limits of pH 7.4 to 7.9, gave identical results. A further experiment was performed, involving the use of large test-tubes for making the final dilution, so that 10 cc. quantities of fluid could be used. In this way it was possible to test the reaction in the suspension tube repeatedly during the course of the experiment. The reaction remained constantly at pH 7.6 to 7.7 and the results agreed with those of the two preceding tests. 0.5 per cent NaCl solution was found to be equally unsuitable as a suspension fluid for pneumococci.

The presence of phosphate was found to have no other than a beneficial effect. Further experiments showed that twice the quantity used in Experiment 3, *i.e.* 1:50, could be used without injury to the pneumococci.

Nature of the Protective Action of Gelatin-Containing Solutions.

In attempting to account for the manner in which the presence of 0.1 per cent gelatin in Locke's solution, salt solution, and water adds so greatly to their protective value as suspension fluids for pneumococci, several possible explanations presented themselves. First to be considered was the possibility that even at room temperature slow growth might occur in a gelatin solution of this concentration. While the presence of growth could hardly account for the difference

found between gelatin-containing and non-gelatin-containing solutions immediately at the completion of the diluting process, yet it might well explain the prolonged life of pneumococci in the former. Accordingly series of plates were made at short intervals of time for periods of 24 to 96 hours on suspensions of pneumococci kept at 22–26°C. and also at 37°C. The results of these tests gave no definite evidence of growth either at incubator or room temperature. There was a progressive diminution in the number of colonies in successive plates, which became marked after 18 to 24 hours. Similar tests were made on suspensions of pneumococci in gelatin solutions of higher gelatin concentrations kept at 37°C. It was not until 0.5 per cent gelatin had been reached that definite evidence of growth was obtained.

Next it was considered possible that agitation of pneumococci in solutions of crystalloids or water might result in injury to the organism and that the action of gelatin might be to protect against such mechanical injury. For Rous and Turner (4) have found that gelatin solutions protect the red blood cells against injury during manipulation.

Experiment 4.—(Table IV.) For this experiment the solutions were buffered with phosphate 1:50 at pH 7.8. A standard suspension was made in gelatin-water and two series of dilutions in water, one in tubes as usual and one in 25 cc. flasks, each of which received 4.5 cc. of solution. At the completion of each flask dilution 0.5 cc. was transferred to the next flask. The tubes of water and gelatin-water were agitated together for the customary lengths of time but rather more vigorously than usual. The flasks were rotated gently, first in one direction and then in the other, for similar lengths of time. Then in order to prevent further injury from agitation the final dilutions of the water series were distributed in 0.1 cc. amounts into a number of small tubes, 1 × 12 cm., each containing 0.9 cc. water, adjusted and buffered as above. This quantity of fluid was sufficient to reduce to a minimum any disturbing effect of evaporation. The sixth dilution of gelatin-water was also distributed in like amounts into tubes containing gelatin-water. Two plates were made at this time from each of the three suspensions. The tubes were kept at a constant temperature of 22–23°C. At varying intervals of time, as indicated in the table, 5 cc. of dextrose blood broth were transferred to one tube of each series, which was then incubated. By this method of culture, any loss of pneumococci which might result from transfer of part of the suspension into the broth, was avoided. Into four tubes of each series, 2 cc. amounts of the water or gelatin-water used above were delivered. These tubes were used for the determination of H ion concentration at intervals during the course of the experiment.

Table IV shows that pneumococci may be so injured by agitation in water that no organisms are recoverable from the suspension by the time the last dilution is completed. The series of dilutions in water carried out by rotating flasks gently exhibited much less injury of the organisms, relatively speaking, as shown by the fact that they could be recovered from suspensions as long as 12 hours afterwards. The contrast, however, between gelatin-water and water (both agitated to the same degree) is striking. The organisms suspended in gelatin-water were alive at the end of 30 hours, and, judging from previous experiments, they would continue to live for several days to a week. The fact that the reaction of the water at 20 hours was pH 8.2, while that of the gelatin-water was pH 8.0, probably had no effect on the

TABLE IV.

Protective Action of Gelatin against Mechanical Injury.

Solution used.	Method employed in making dilutions.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs.									pH of solution. At hrs.			
			0	1	3	6	9	12	20	24	30	0	5	20	30
Water.	Agitation in tube.	0	0	0	0	0	0	0	0	0		7.5	7.7	8.2	8.0
"	Rotation in flask.	16	+	+	+	0	0	+	0	0		7.5	7.7	8.2	8.0
Gel.-water.	Agitation in tube.	53	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9

outcome of the experiment, since it has been found that pneumococci survive well in suspension fluids with a reaction as alkaline as pH 8.2. An H ion concentration, however, of pH 8.3 to 8.4 seems to be definitely unfavorable. In a repetition of the experiment, in which the reaction remained constant at pH 7.9 to 8.0 in both the flask and tube dilutions, organisms were recovered from the tube only immediately at the completion of the dilutions, in broth but not in plate, while three plates from the flask gave an average of 51 colonies (34, 52, 68, respectively) and positive broth cultures were obtained from the suspension for 3 hours. The degree of protection afforded by the presence of gelatin in water seems to be very great, since even the most vigorous shaking that could be given by striking the tube against the hand did not apparently injure the pneumococci.

From the results of the above experiment, which was repeated several times, it seemed not improbable that the whole effect of gelatin was to protect against mechanical injury; and that in the absence of agitation, solutions without gelatin might prove as suitable suspension fluids as those containing gelatin, with the possible exception of salt solutions. In order to test this possibility an experiment was devised whereby a suspension containing small numbers of pneumococci prepared with as little injury as possible, could be delivered into a series of solutions with and without gelatin, for comparison of time of viability.

TABLE V.

Preservative Action of Gelatin Solutions.

Solution used.	Per cent of gelatin in inoculum fluid.	Final per cent of gelatin in suspension fluid.	Growth in dextrose blood broth.									pH of solution.			
			At hrs.									At hrs.			
			0	1	3	6	9	12	20	24	30	0	5	20	30
	per cent	*per cent*													
Water.	0.001	0.0001	+	+	+	+	+	0	0	+	0	7.5	7.7	8.2	8.0
Gel.-water.	"	0.1	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9
Salt sol.	"	0.0001	+	+	+	0	0	0	0	0	0	7.6	7.9	8.2	8.2
Gel.-salt.	"	0.1	+	+	+	+	+	+	+	+	0	7.7	7.9	8.2	8.2
Locke's sol.	"	0.0001	+	+	+	+	+	0	0	0	0	7.1	7.6	7.7	7.7
Gel.-Locke's.	"	0.1	+	+	+	+	+	+	0	0	0	7.3	7.5	8.0	8.0
Water.	0.01	0.001	+	+	+	+	+	+	0	0	0	7.5	7.7	8.2	8.0
Gel.-water.	"	0.1	+	+	+	+	+	+	+	+	+	7.4	7.5	8.0	7.9
Salt sol.	"	0.001	+	+	+	+	+	0	0	0	0	7.6	7.9	8.2	8.2
Gel.-salt.	"	0.1	+	+	+	+	+	+	+	+	+	7.7	7.9	8.2	8.2
Locke's sol.	"	0.001	+	+	+	+	+	0	0	0	0	7.1	7.6	7.7	7.7
Gel.-Locke's.	"	0.1	+	+	+	+	+	+	0	+	+	7.3	7.5	8.0	8.0

Experiment 5.—(Table V.) Solutions prepared as in Experiment 4. Standard solution and main series of dilutions made in gelatin-water. In order to diminish as much as possible the amount of gelatin in the final dilution to be used for the inoculation, and at the same time to reduce to a minimum the injury which the pneumococci undergo when agitated in plain water, 0.1 cc. of the fourth dilution in the gelatin-water series was transferred into 9.9 cc. of plain water contained in large test-tubes 25 mm. × 20 cm. In this way the sixth dilution was reached with only one period of agitation in water and this contained 0.001 per cent of gelatin. As a control, a second dilution in plain water was made from the fifth dilution of the gelatin water series in the usual way, since it was feared that a

single agitation in water containing as little gelatin as 0.001 per cent might cause injury to the organisms. These two suspensions were delivered in 0.1 cc. quantities into a number of small tubes 1 × 12 cm. each containing 0.9 cc. of the solution to be tested. Half way through this process five plates were made from one of the suspensions and four from the other. Successive tenths of a cc. from the 0.1 to 0.9 marks on the pipette were used. The same pipette was used throughout with each suspension. Also into four tubes of each series 2 cc. of the solution were delivered for purposes of testing the H ion concentration at intervals during the test. The tubes were kept at a temperature which remained constantly between 22 and 23°C. At successive periods of time, indicated in the table, 5 cc. dextrose blood broth were transferred into one tube of each series, which was then incubated.

Table V shows that in every instance the pneumococci survived for a longer period of time in the gelatin-containing solutions than in those containing no gelatin. This difference was most marked in the case of salt solution. The number of organisms in the inoculum was about that employed in the previous experiments; the nine plates showed from 38 to 62 colonies—an average of 52. A repetition of a part of the experiment with water, gelatin-water, salt solution, and gelatin-salt, gave essentially the same results. In this latter experiment the test was run for a longer period, and the difference in the survival time between water and gelatin-water was considerably greater. No organisms could be recovered from the water after 6 hours, while in the gelatin-water, organisms were still found to be viable at 72 hours. (The H ion concentration of the solutions remained more constant—between pH 7.4 and 7.9 throughout the entire test.) Since the organisms implanted in the various test solutions were in the same state and approximately in the same numbers to begin with, and the factor of mechanical injury as a variant was entirely excluded, it seems fair to conclude that the longer period of survival in the gelatin-containing solutions indicates a definite preservative or maintaining action of gelatin for pneumococcus, in addition to the protection it affords against mechanical injury. The term preservative is used simply to indicate a second distinct property of the gelatin solutions, which should be properly included under the general designation of protective action, since the prolonged life of the organisms indicates protection against those influences causing early death in the non-gelatin-containing solutions.

It should further be pointed out that in the first part of the test in which the inoculum fluid contained a very small amount of gelatin, the pneumococci lived a much shorter length of time in salt solution than in either Locke's solution or water. This has been found in subsequent tests, and corroborates Shearer's findings regarding the toxicity of NaCl solutions for pneumococci.

The Beneficial Effect of Phosphate in Gelatin-Salt 0.9 Per Cent Solution.

While the results of the foregoing experiments seemed to demonstrate that the addition of 0.1 per cent gelatin to 0.9 per cent NaCl solution produces a suitable fluid for suspension of pneumococci, attempts to utilize this solution as the medium for suspension and dilution in certain experiments on growth inhibition, showed clearly that the organisms were markedly injured during their sojourn in the gelatin-salt solution. A resumé of the protocols of the tests on gelatin-salt showed that in each instance in which gelatin-salt 0.9 per cent was used, the main series of dilutions had been made up in either gelatin-salt 0.5 per cent or gelatin-water, and that the dilutions in gelatin-salt 0.9 per cent were made from the fourth or fifth dilutions of this main series. Furthermore, in every experiment in which gelatin-salt 0.9 per cent showed well marked protective properties for pneumococci, phosphate was present in the solution. The gelatin-salt solution used for the pneumococcus suspension in the above mentioned experiments on growth inhibition did not contain phosphate. It seemed probable, then, that either the greater length of time of agitation in gelatin-salt 0.9 per cent solution or the absence of phosphate would account for the lack of protection shown. In the following experiment the effect of each of these two factors was tested.

Experiment 6.—(Table VI.) The standard suspension was made up in gelatin-salt 0.5 per cent, and one series of dilutions made in this same fluid. The gelatin-salt 0.9 per cent series, with and without phosphate, were each begun from the standard suspension. The phosphate-containing solutions were adjusted and buffered by first adding 1:50 H_3PO_4 then NaOH to the desired reaction.

One series of dilutions was made in gelatin-water (containing phosphate), from the fourth dilution of which the series was continued in gelatin-salt 0.9 per cent (indicated in the table). The sixth dilution of the above suspensions was made in 10 cc. quantities (in 50 cc. test-tubes) and kept at a temperature of

20–22°C. At intervals, the tubes were agitated 3 to 4 minutes, and 0.1 cc. of the contents transplanted into 5 cc. dextrose blood broth. Also 2 cc. quantities of the suspension were removed from time to time for testing the H ion concentration. The last two gelatin-salt 0.9 per cent suspensions shown in the table were put up at a different time, and from another gelatin-water series of dilutions. The suspension was distributed into small test-tubes, into which dextrose blood broth was transferred at intervals, as previously described.

TABLE VI.

Beneficial Effect of Phosphate in Gelatin-Salt 0.9 Per Cent Solution.

Solution used.	Amount of phosphate.	No. of colonies in plate.	Culture in dextrose blood broth. At hrs.										pH of suspension. At hrs.			
			0	2	4	6	9	11	21	24	30	44	0	2	4	21
per cent																
Gel.-salt 0.5.	1:50	32	+	+	+	+	−	+	+	−	+	+	7.0	7.1	7.3	7.5
" " 0.9.	1:50	22	+	+	+	+	−	+	+	−	0	0	6.8–7.0	7.1	7.3	7.5
" " 0.9.	0	76	+	+	+	+	−	0	0	−	0	0	6.8–7.0	7.2	7.4	7.4
" " 0.9. From 4th dil. of gel.-water.	1:50	25	+	+	+	+	−	0*	+	−	0	0	7.2	7.3	7.4	7.5
Gel.-salt 0.9. From 5th dil. of gel.-water.	1:50		+	+	+	+	+	−	+	+	0	0	7.4†		7.5	7.6
Gel.-salt 0.9. From 5th dil. of gel.-water.	0		+	+	+	+	0	−	0	0	0	0	7.6†		7.7	7.9

* Culture tube contaminated.

† The pH of the final dilution tubes was 7.0 in the phosphate-containing, and 6.9 in the non-phosphate-containing suspensions.

The results of this experiment (Table VI) show clearly that the protective action of gelatin-salt 0.9 per cent depends very largely on the presence of the phosphate. In the absence of phosphate the pneumococci survived for only a brief period, regardless of whether agitation had been long or short. Additional experiments with varying concentrations of NaCl in gelatin-salt solution (containing phosphate) showed that in solutions of 0.6 per cent or 0.5 per cent NaCl, the survival time was definitely longer than when a higher percentage of NaCl was employed. In comparing gelatin-salt 0.9 per cent with gelatin-salt 0.5 per cent, both without phosphate, it

was found that pneumococci survived for a very much longer time in the latter solution—as long as 48 hours—but even gelatin-salt 0.5 per cent showed a definite increase in its protective properties after the addition of phosphate.

Certain other findings may be briefly summarized as follows: The protective action of gelatin for pneumococci suspended in non-nutrient fluids was found not to be peculiar to this substance alone. Serum added to salt solution, Locke's solution, or water in an amount such that the weight of the solids in the serum equalled 0.1 per cent of the solution, was found to exert a protective effect similar to that of gelatin. In attempting to account for the way in which gelatin protected pneumococci against the toxic action of NaCl, especially in 0.5 per cent solution without phosphate, the gelatin used was analyzed for its calcium content. This was found to be 38 mg. of Ca per 100 gm. of gelatin. The gelatin was now purified by the method of Loeb (5) and a reanalysis showed a calcium content of only 2.5 mg. per 100 gm. of gelatin. Pneumococci suspended in gelatin-salt 0.5 per cent made with this purified gelatin showed a survival period but slightly shorter than that which occurred in the non-purified gelatin-salt of the same per cent. Hence the beneficial effect of gelatin in salt solution of this concentration could not be accounted for, to but a small degree only, by the calcium content of the gelatin used.

DISCUSSION.

While the effect of shaking on certain other microorganisms has been studied, we have not been able to find any mention in the literature of the effect of agitation on the pneumococcus. Meltzer (6) observed many years ago that *Bacillus megaterium* could be injured by continuous shaking in both water and salt solution for a number of hours, but the effects were frequently not marked. *Bacillus subtilis* and a "yellow coccus from the air" were found by him to be even less susceptible to injury by shaking. Appel (7) repeated Meltzer's work and found that the bacteria could be injured only by violent agitation in tubes containing glass beads. Our results show that pneumococci, on the contrary, are markedly injured by a relatively brief period of moderately vigorous agitation when suspended in water or fluids containing only crystalloids.

The mechanism of the protective action of gelatin for pneumococci is not clear. Protection against mechanical injury may conceivably be due to the presence of the large gelatin molecules, acting to diminish the impact of collision between individual organisms and between organisms and the glass walls. It is possible that the gelatin may even form a protective coating around the individual pneumococci. Concerning the nature of the further protective effect of gelatin for pneumococci under static conditions or its preservative action, several possibilities present themselves. (1) The presence of gelatin in the solution may cause a retardation of autolysis by the enzymes of the bacterial cell. Lord and Nye (8) have put forth this theory to account for the longer life of pneumococci suspended in serum, as contrasted with similar suspensions in solutions of crystalloids. (2) Colloids such as gelatin may act to prevent penetration of the bacterial cell wall by the surrounding fluid—a process that, occurring in non-protein-containing solutions, may ultimately lead to death of the organism. (3) It is further possible that in protein-containing solutions pneumococci are enabled to carry on certain metabolic processes despite the absence of growth, which tend to prolong the life of the organism. (4) A very slow multiplication of the organisms, though unlikely, cannot be excluded, despite the results of the plating experiments which show after a certain number of hours a gradual and progressive diminution in pneumococci recovered from 0.1 per cent gelatin solutions kept at a temperature of 22–23°C.

The manner in which 0.1 per cent gelatin in 0.5 per cent NaCl solution protects the pneumococci against injury by the Na ion remains unexplained. The additional protective action of phosphate, however, in the higher concentrations of NaCl (in gel.-salt) suggests an antagonism between sodium phosphate and sodium chloride. In the last experiment described and others summarized there was no potassium present, since Na_2HPO_4 alone was used as buffer. While the study of antagonistic salt action has shown that the cations are chiefly concerned in this phenomenon, yet certain instances have been found in which anions played a definite rôle. Loeb (9) found that Na acetate was much more effective than NaCl in protecting the eggs of fundulus against the toxic action of KCl. Brooks (10) studying the action of various ions on the respiration of

Bacillus subtilis, showed that the depressant effect of NaCl could be neutralized by Na taurocholate. Further study is in progress to determine whether or not there exists a true antagonism between NaCl and Na_2HPO_4.

The presence of 0.1 per cent gelatin in the fluid used for the pneumococcus suspension, has been found not to interfere with phagocytosis or intracellular digestion of the pneumococci. Its effect on other immunological reactions has not been tested.

It seems not improbable that pathogenic microorganisms, as a class, are more susceptible to mechanical injury than is commonly supposed. In numerous bacteriological and immunological procedures, high dilution of the bacterial suspension used is required. In certain of these tests vigorous and often prolonged agitation is employed in making the dilutions. Even though sterility of the suspension is not produced by this process, the organisms may be so injured as to interfere seriously with growth. In view of the findings brought out by the study of pneumococcus suspensions, it may be found advantageous to employ gelatin more widely in solutions used for the suspension of bacteria.

SUMMARY.

Experiments were undertaken with the purpose of determining the type of non-nutrient fluid most suitable for the suspension of pneumococci with a minimum amount of injury. For comparative studies, dilutions in the various fluids tested were made from a standard suspension containing approximately 1,000 million pneumococci per cc. The protective properties of each solution were judged by the length of time viable organisms could be recovered (in culture) from a suspension containing 0.0000001 cc. of the standard suspension. The hydrogen ion concentration of the solutions, the temperature at which the suspensions were kept, and the culture media were carefully controlled. Furthermore, plates were made at the beginning of the experiment to determine the number of organisms present in a unit of suspension.

It was found that pneumococci suspended in Locke's solution, 0.5 and 0.9 per cent NaCl solutions, and water, remained alive for only

a few hours at most. Salt solution was shown to be the least suitable of these three; not infrequently by the time the 0.000001 cc. dilution in this fluid had been reached, the suspension was sterile. The addition, however, of 0.1 per cent gelatin to the above fluids transformed all, except 0.9 per cent NaCl, into highly favorable solutions. Pneumococci suspended in gelatin-water and gelatin-Locke's solution remained alive at room temperature for 6 to 7 days; in gelatin-salt 0.5 per cent solution, for at least 2 days. It was possible to vary the H ion concentration of the suspension fluids from pH 7.0 to 8.2 without any marked effect on the results. However, a pH of 7.4 to 8.0 appeared to be the most suitable.

The nature of the protective action of gelatin was investigated. Series of plates made at frequent intervals failed to reveal any growth in suspensions of pneumococci containing this small concentration of gelatin. The beneficial effect of gelatin in the above solutions was found to lie largely in its protection of the pneumococci against the mechanical injury which occurs during the process of dilution in crystalloid solutions or water. Gelatin shows in addition a well marked preservative action, that is to say, it protected organisms against early dissolution. The nature of this preservation is uncertain.

The presence of 0.1 per cent gelatin in 0.9 per cent NaCl failed to protect pneumococci against the toxic action of Na. But it was found possible to neutralize to a considerable degree this toxic effect of sodium by the addition of a small quantity (2 per cent) of a M/15 balanced phosphate mixture or sodium phosphate. In a gelatin-salt 0.9 per cent solution buffered with phosphate, pneumococci survived for 24 to 48 hours. Even in gelatin-salt, 0.5 per cent the presence of phosphate had a definite beneficial effect.

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